AD_____

Award Number: DAMD17-97-1-7116

TITLE: Contrast Enhanced 3D Color Amplitude Imaging of the Breasts

PRINCIPAL INVESTIGATOR: Flemming Forsberg, Ph.D.

CONTRACTING ORGANIZATION: Thomas Jefferson University
Philadelphia, Pennsylvania 19107

REPORT DATE: October 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1999	Annual (15 Sep 98 - 14 Sep 99)

4. TITLE AND SUBTITLE
Contrast Enhanced 3D Color Amplitude Imaging of the Breasts

5. FUNDING NUMBERS
DAMD17-97-1-7116

6. AUTHOR(S)
Flemming Forsberg, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Thomas Jefferson University
Philadelphia, Pennsylvania 19107

E-MAIL:
forsberg@esther.rad.tju.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

In total, 22 patients with 6 cancers and 16 benign lesions have been enrolled in the Levovist arm of the study. These numbers were less than anticipated, and an initial data analysis indicated that Levovist was not as efficacious as expected. To increase recruitment and improve results the ultrasound contrast agent was changed to Optison. To date 13 patients with 3 cancers and 10 benign lesions have been enrolled in this part of the study. Moreover, 3D parameter extraction algorithms for the LIS 6000A system has been designed and tested, but no data analysis has yet been carried out due to the limited data set available for Optison.

The histomorphometry system has been used to analyze vessel distribution and vessel density maps from 10 patients. Contrast enhanced color flow imaging was found to provide some quantitative parameters, which correlated with direct pathologic vascularity assessments such as the iMVD. Specifically, the microvessel area and count for vessels 30 to 39 μm in diameter were most significant. These results indicate that ultrasound imaging with contrast may produce a quantitative measure of the neovascularity within breast tumors. However, the current patient population in the sub-study is very small and further cases are currently being analyzed.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	33
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

X Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

___ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

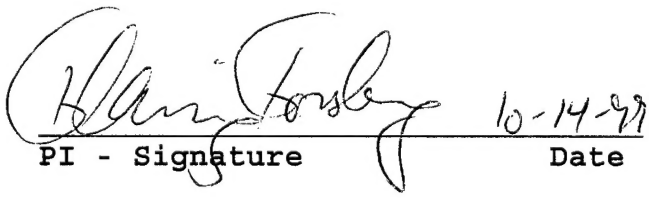
N/A In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

X For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.



PI - Signature 10-14-99 Date

4. TABLE OF CONTENTS

1. FRONT COVER.....1

2. SF 298.....2

3. FOREWORD.....3

4. TABLE OF CONTENTS.....4

5. INTRODUCTION.....5

6. BODY.....6

    6.1 Methods 6

    6.2 Results and Discussion 8

7. KEY RESEARCH ACCOMPLISHMENTS.....9

8. REPORTABLE OUTCOMES.....10

9. CONCLUSIONS.....10

10. REFERENCES.....11

11. TABLES.....12

12. CAPTIONS.....14

13. APPENDICES.....16

5. INTRODUCTION

The goal of any breast imaging modality is to improve the early detection of tumors and to improve the differentiation between benign and malignant lesions. While x-ray mammography is efficacious in diagnosing a high percentage of breast masses, it also produces a high rate of false positives [1]. The percentage of breast biopsies that are actually malignant vary between 10 % and 35 %. Thus, a technique which reliably differentiates between malignant and benign masses would improve the diagnosis of breast cancer and should, therefore, reduce the number of negative biopsies as well as the trauma of the patients. This project attempts to establish such a technique through the novel and innovative combination of three-dimensional (3D) ultrasound imaging with a contrast agent.

Ultrasound imaging is currently an auxiliary modality in breast imaging. It is mainly used to differentiate between cystic and solid lesions [2]. Investigations into the possibility of breast cancer diagnosis based on Doppler ultrasound flow detection have produced mixed results, due to overlap between flow measurements in benign and malignant tumors [3-4]. One problem may be the lack of sensitivity in flow detection in small tumor vessels using ultrasound. This hypothesis is supported by reports in the pathology literature describing angiogenic vascular morphology as an independent predictor of metastatic disease [5].

Ultrasound contrast agents produce increases of 15 to 25 dB in the echo intensities of blood flow signals; especially when combined with a new display technique called color amplitude imaging (CAI) [6-7]. Thus, an interesting research study can be devised, which compare the ability of two-dimensional (2D) color Doppler ultrasound with and without contrast to differentiate between benign and malignant masses relative to x-ray mammography. This is in essence the purpose of a 5 year National Institutes of Health (NIH) funded project recently awarded to Thomas Jefferson University. The current project (DAMD17-97-1-7116) is an expansion to the NIH project which adds 3D flow imaging with and without contrast, since 3D imaging should be better suited than 2D ultrasound to demonstrate the tortuous angiogenic vasculature associated with breast cancer.

Not only is the potential of the novel combination of 3D imaging and contrast in itself innovative, but because of the NIH funded study it will be possible to compare a number of new and unique approaches to breast cancer diagnosis i.e., 2D and 3D CAI with and without contrast as well as harmonic imaging directly to x-ray mammography. Furthermore, this project is extremely cost-effective because the NIH grant coves a majority of the personnel costs as well as all major equipment purchases. The amalgamation of the NIH project with the current proposal also allows for basic research into the correlation between Doppler flow signals and pathologically detected lesion vascularity. This will enable a deeper understanding of the relationship between tumor neovascularity and ultrasound flow measurements; again at very little cost to this proposal.

Consequently, this project is an add-on study to the already funded NIH project aimed at increasing the sensitivity and specificity of breast ultrasound by combining injection of an ultrasound contrast agent with 3D reconstruction of color amplitude images. The fundamental

hypothesis is that the neovasculature of malignant lesions can be visualized with this novel combination, thus, improving the diagnosis of breast cancer.

6. BODY

The central hypothesis of this project is that the differentiation between benign and malignant breast lesions can be improved by visualization of tumor neovascularity using 3D ultrasound imaging in conjunction with an ultrasound contrast agent. To investigate this hypothesis 150 women with breast lesions will be recruited over three years and imaged using contrast enhanced 3D CAI. The specific tasks of the project (as presented in the original Statement of Work) can be found in Appendix I.

First an outline of the methods applied will be given followed by a presentation of the results to date. Finally, the conclusions and future directions of the research will be discussed.

6.1 Methods

The 3D CAI reconstructions in this project are performed with a state-of-the-art ultrasound scanner connected to an LIS 6000A 3D Image Acquisition and Reconstruction system (Life Imaging Systems Inc, London, Canada). To date all 3D CAI acquisitions have been performed with an HDI 3000 scanner (Advanced Technology Laboratories, Bothell, WA). The 3D CAI data sets are reconstructed, with no loss of registration accuracy, to provide both multi-perspective Maximum-Intensity-Projection visualization and 2D planar views.

The patients used in this project will be women of a wide variety of ages having a breast mass or abnormality resulting in a breast excisional biopsy. Breast cancer in males accounts for only about 1 % of cases in our hospital and thus, were not be included in the patient population. All patients will be referred after X-ray mammography identifies a mass or suspicious area. The target enrollment is approximately 50 patients per year, which represents half of the patients being recruited for the NIH-supported study.

Following a baseline ultrasound gray scale scan, which identifies the mass seen by x-ray mammography, images for 3D CAI of the lesion are acquired. Next, an ultrasound contrast agent is administered intravenously via a peripheral vein, preferably the antecubital vein. An initial videotaped sweep of the mass will be made with the gain and CAI settings unaltered from the pre contrast settings. This will allow for side by side comparison of pre and post contrast CAI studies. Following this, the CAI settings will be optimized for the stronger contrast enhanced Doppler signals. As the SNR improves it should be possible to achieve higher frame rates and/or line density, improving spatial resolution of the color. Videotaped sweeps of the abnormality will be made every 1 to 2 minutes through the period of enhancement (up to 6 minutes). A second injection is made to acquire 3D CAI data with the gain and other settings unaltered from the pre contrast protocol. This will allow for side by side comparison of pre and post contrast 3D CAI studies. Next the CAI settings will be optimized for the stronger contrast enhanced signal, and 3D data will be acquired again.

The ultrasound findings will be correlated to the pathology sections and the radiologist and pathologist will attempt to correlate the findings by each method. If the ultrasound and pathology sections do not match it is possible in the 3D case to resection the acquired volume until a match is found. After removal of the mass, the specimens will be sectioned in the same plane as the ultrasound images and stained with an endothelial cell marker, CD31, which targets the microvessel walls staining them brown to dark brown in color. Finally, the sections will be mounted onto 2"x 3" glass slides. The vascular morphology of the tissue, specifically, the number and area occupied by tumor vessels, will be determined by a semi-automated histomorphometry system based on SMZ-10A microscope (Nikon, Melville, NJ) and ImagePro Plus software (Media Cybernetics, Silver Spring, MD). Using the software, microscope, and a Sony CCD camera, the entire specimen area is captured and digitized under 100x magnification. The frame size captured is equal to 640 pixels by 480 pixels or 1.27 mm^2. The number of color pixels on the color flow images relative to the pixel size of the mass can be used as a first order measure of mass vascularity [8]. Frozen ultrasound images will be captured before and after contrast injection and digitized using the image analysis software.

Both the slide and video image analyses are patterned after chromaticity analysis methods used by Barbareschi et al. and Bell et al., respectively [9-10]. The aim of the slide image analysis is to develop a method in which only microvessels with lumen would be recognized by the software and, hence, counted and measured. Only vessels with lumen will be chosen for three reasons: 1) the diameter of the vessels can be assessed directly, 2) other structures sporadically stained will not give false positives, and 3) a more repeatable and automated method can be performed. For each captured RGB color image of tumor area the slide image processing consists of extracting vessel (saturation image) and tissue (blue image) enhanced images and performing mathematical morphometry to obtain an image on which automated count and measurements of microvessels was performed. For each slide the total microvessel area (MVA) and count (MVC) will be determined and divided into five categories: vessel diameters between a) 10-19 μm, b) 20-29 μm, c) 30-39 μm, d) 40-49 μm, and e) 50 μm and above. The ultrasonic image processing consists of extracting red, green, and blue images and performing mathematical morphometry to obtain an image only with color pixels. From the video analysis, the percent of color pixels within the mass will be calculated for each level before and after contrast administration.

To determine if a linear relationship existed between pathologic and ultrasonic vascularity measurements, an equal number of data points must be assessed from both methods. Ten ultrasonic data points per patient (5 pre and 5 post contrast) will consistently be obtained because a hand held transducer is used and regardless of the size of the mass, scans at five levels of each mass can always be taken (albeit, sometimes with overlap between the levels). However, due to the size of some masses, five pathologic sections of a mass will sometimes not be possible. Therefore, to be able to compare the data sets two solutions were developed, which ensures equal number of points in both data sets. One solution requires ultrasonic data to be averaged and replaced until for each patient the number of scans equals the number of pathology slides (i.e., data reduction). The other solution requires pathologic data to be averaged and added to the number of slides for each patient until five data points were reached (i.e., data expansion). Statistical analysis is performed to determine if ultrasonic flow data (pre or post contrast)

correlated with pathologic data in breast tumors. The linear relationship between ultrasonic and pathologic data will be assessed using single and multiple variable linear regression techniques.

6.2 Results and Discussion

Between January 1998 and January 1999, 22 patients (with 6 cancers and 16 benign lesions) have been enrolled in the project and studied using contrast enhanced 3D CAI with Levovist. Table 1 provides a summary of the additional two patients recruited in Year 2. These numbers were less than anticipated, due mainly to difficulties in enrolling patients into a 4 hour ultrasound contrast agent study immediately prior to their breast surgery. An initial data analysis, as envisaged under task 3, has been completed and it indicated that Levovist was not as efficacious as expected. Technical failures eliminated four cases. Of the remaining 18 lesions, 4 were cancers, 12 were benign and 2 were proliferative lesions. There was a marked increase in vascularity seen pre to post contrast with both 2D and 3D US ($p < 0.01$). The diagnostic accuracy of 3D power Doppler was 56 % (10 correct diagnoses out of 18 studies), which was somewhat better than 2D (44 % or 8 out of 18). Sensitivity and specificity were 25 % and 58 % for 3D US, while for 2D US these ratios were 50 % and 42 %, respectively. Contrast changed three diagnoses in 3D mode (2 of which improved diagnostic confidence). In 2D US the addition of contrast produced five changes in the final diagnosis (3 were improvements). These results are somewhat disappointing, especially as there was no statistically significant improvement from pre to post contrast imaging.

More importantly, an interim analysis of all the 79 patients recruited in the NIH funded study also lead us to doubt the efficacy of Levovist. Consequently, it was decided to change the ultrasound contrast agent to Optison® (Molecular Biosystems Inc., San Diego, CA), which is a third generation agent (unlike Levovist) and is approved by the FDA for use in echocardiography. Optison will be administered intravenously via a peripheral vein, preferably into the antecubital vein. Dosages from 0.5 to 4.0 ml will be given with the total dose not exceeding 8.7 ml. These dosages typically provide up to 5 minutes of enhancement. Optison is well tolerated by patients and safety issues related to the agent are not a concern in this study.

This decision led to a four month stop in patient recruitment, while the associated protocol changes were implemented. As an added benefit the examination time was reduced from 4 hours to 1 hour, since less safety data is required for a contrast agent already approved for another indication. Moreover, Molecular Biosystems have agreed to supply the agent free of charge. From May of 1999 and until September 13 patients with 3 cancers and 10 benign lesions have been enrolled in the Optison arm of the study (Table 2). The patients had a mean age of 53 years (range 34 to 72 years). When accounting for the occasional hardware problems with the LIS 600A unit there is clearly an increase in the recruitment rate relative to Year 1. Finally, the 3D parameter extraction algorithms for the LIS 6000A system has been designed and tested, but no data analysis has yet been carried out due to the limited data set available for Optison. In summary, task 1 has been completed and tasks 2 and 3 are ongoing.

A subset of 10 patients with 31 pathology slides (for a total of 5756 frames) were examined with the histomorphometry system. A total of 100 digitized ultrasound scans (5 pre and 5 post contrast for each patient) were included in this part of the study. Figure 1 shows sample

digitized ultrasonic and pathologic images for both a benign and a malignant breast mass. There was a significant increase in the number of ultrasound color pixels pre to post contrast injection ($p < 0.003$). No statistical difference in ultrasonic color pixels was found between the benign and malignant masses. Table 3 gives the r^2 values for the linear regression for the reduced and expanded data sets. The multiple linear regression technique for the entire data set (i.e., benign and malignant lesions evaluated jointly) found significant correlations for ultrasonic color pixels post injection with the percent of area in the five vessel ranges (expanded MVA; $p = 0.02$) and with the percent of vessels in the five vessel ranges (reduced iMVD; $p = 0.06$). No correlations were found between pre contrast ultrasonic color pixels and pathologic vascularity measurements using the entire data set.

To determine which variable in the multiple linear regression contributed the most, the T-statistic was evaluated for each variable. The significant T-values are listed for the reduced and expanded data sets in Tables 4 and 5, respectivley. For both the percent of area and percent of vessels in the five vessel ranges, the 30 to 39 μm vessel range contributed the most significantly to the linear relationship with the percent of color pixels post contrast injection, ($p = 0.004$ expanded and $p = 0.005$ reduced, respectively). Note, that the pre injection ultrasound vascularity measures from benign masses have the most significant contributions from larger vessels (≥ 40 μm) than the post injection ones (Table 4).

From the statistical analysis, it can be inferred that contrast enhanced color flow imaging provides some quantitative parameters, which correlate with direct pathologic vascularity assessments such as the iMVD. Specifically, the microvessel area and count for vessels 30 to 39 μm in diameter were most significant. This is in agreement with the qualitative observations of Burns et al. [11]. These results indicate that ultrasound imaging with contrast may produce a quantitative measure of the neovascularity within breast tumors; similar to results recently obtained with MRI [12]. However, the current patient population is very small and until further patients are analyzed, these conclusions are preliminary.

Given the problems encountered in the patient population that received Levovist, as described above, no publications have been produced from that patient population in Year 2. However, the quantitative sub-study correlating contrast enhanced ultrasound and pathology has been presented (see section 8) and submitted to the annual meeting of the AIUM [13]. Results are currently in press in a peer-reviewed journal [14] (see also Appendix II).

1. KEY RESEARCH ACCOMPLISHMENTS

- In total 35 patients studied using contrast enhanced 3D CAI.
- Marked increase in vascularity seen pre to post contrast imaging ($p < 0.01$).
- Contrast enhanced color flow imaging provides some quantitative parameters, which correlate with direct pathologic vascularity assessments.
- Contrast enhanced ultrasound images of breast tumor vascularity correspond to vessels 30 to 39 μm in diameter ($p = 0.02$ and 0.06).

2. REPORTABLE OUTCOMES

Manuscripts, abstracts, presentations

Forsberg F, Chaudhari MH, Voodarla A, Goonewardene S, Needleman L, Goldberg BB: Qunatifying breast tumor neovascularity by contrast enhanced ultrasound and pathology: a comparative study. Submitted to *AIUM*, 2000.

Chaudhari MH, Forsberg F, Voodarla A, Saikali FN, Goonewardene S, Needleman L, Shi WT, Finkel GC, Goldberg BB: Breast tumor vascularity identified by contrast enhanced ultrasound and pathology: initial results. *Ultrasonics*, 38, 2000. In press.

June 29 - July 1, 1999. Ultrasonics International '99, Copenhagen, Denmark.

- Breast tumor vascularity identified by contrast enhanced ultrasound and pathology: initial results.

Degrees

A Master of Science degree in Biomedical Engineering was awarded from Drexel University to Manisha H. Chaudhari based in part on work performed as part of this grant. The PI (F. Forsberg) served as her supervisor.

9. CONCLUSIONS

In total, 22 patients with 6 cancers and 16 benign lesions have been enrolled in the Levovist arm of the study. These numbers were less than anticipated, and an initial data analysis indicated that Levovist was not as efficacious as expected. To increase recruitment and improve results the ultrasound contrast agent was changed to Optison. To date 13 patients with 3 cancers and 10 benign lesions have been enrolled in this part of the study. Moreover, 3D parameter extraction algorithms for the LIS 6000A system has been designed and tested, but no data analysis has yet been carried out due to the limited data set available for Optison.

The histomorphometry system has been used to analyze vessel distribution and vessel density maps from 10 patients. Contrast enhanced color flow imaging was found to provide some quantitative parameters, which correlated with direct pathologic vascularity assessments such as the iMVD. Specifically, the microvessel area and count for vessels 30 to 39 μm in diameter were most significant. These results indicate that ultrasound imaging with contrast may produce a quantitative measure of the neovascularity within breast tumors. However, the current patient population in the sub-study is very small and further cases are currently being analyzed.

In summary, task 1 has been completed and tasks 2 and 3 are ongoing.

10. REFERENCES

1. Feig SA: Breast masses: Mammographic and sonographic evaluation. *Radiologic Clin North Am* 30:67-92, 1992.
2. Jackson VP: The Role of US in Breast Imaging. *Radiology*, 177:305-311, 1990.
3. Bohm-Velez M, Mendelson EB: Computed tomography, duplex Doppler ultrasound and magnetic resonance imaging in evaluating the breast. *Semin Ultrasound CT MR*, 10:171-176, 1989
4. Adler DD, Carson PL,. Rubin JM, Quinn-Reid D: Doppler ultrasound color flow imaging in the study of breast cancer: preliminary findings. *Ultrasound Med. Biol.*, 16: 553-559, 1990.
5. Weidner N, Folkman J, Pozza F, et al. Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast cancer. *J Natl. Cancer Inst.,* 84: 1875-1887, 1992.
6. Goldberg BB. *Ultrasound contrast agents*. Martin Dunitz Ltd., London, UK, 1997.
7. Forsberg F, Merton DA, Liu JB, Needleman L, Goldberg BB: Clinical applications of ultrasound contrast agents. *Ultrasonics*, 36: 695 - 701, 1998.
8. Meyerowitz CB, Fleischer AC, Pickens DR, et al. Quantification of tumor vascularity and flow with amplitude color Doppler sonography in an experimental model: preliminary results. *J Ultrasound Med.* 15:827-?, 1996.
9. Barbareschi M, Weidner N, et al. Microvessel density quantification in breast carcinomas. *Appl. Immunohistochem.* 3:75-?, 1995.
10. Bell D, Bamber J, Eckersley R. Segmentation and analysis of color Doppler images of tumor vasculature. *Ultrasound Med. Biol.* 21:635 -?, 1995.
11. Burns P, Powers J, Simpson D, Brezina A, Kolin A, Chin C, Uhlendorf V, Fritzch T. Harmonic power mode Doppler using microbubble contrast agents: an improved method for small vessel flow imaging. *Proc IEEE Ultrason. Symp*, 1547-1550, 1994.
12. Esserman L, Hylton N, George T, Weidner N. Contrast-enhanced magnetic resonance imaging to assess tumor histopathology and angiogenesis in breast carcinoma. *The Breast J.* 5:13-21, 1999
13. Forsberg F, Chaudhari MH, Voodarla A, Goonewardene S, Needleman L, Goldberg BB: Qunatifying breast tumor neovascularity by contrast enhanced ultrasound and pathology: a comparative study. Submitted to *AIUM*, 2000.
14. Chaudhari MH, Forsberg F, Voodarla A, Saikali FN, Goonewardene S, Needleman L, Shi WT, Finkel GC, Goldberg BB: Breast tumor vascularity identified by contrast enhanced ultrasound and pathology: initial results. *Ultrasonics*, 38, 2000. In press.

11. TABLES

Table 1: Enrollment details for Levovist patients.

Patient no.	age [years]	race	diagnosis
1076	58	Caucasian	Fibrocystic Changes
1079	43	Caucasian	Atypical Lobular Hyperplasia

Table 2: Enrollment details for Optison patients.

Patient no.	age [years]	race	Diagnosis
1080	47	Caucasian	Proliferative Fibrocystic Changes
1081	53	Black	Stromal fibrosis
1082	43	Caucasian	Intraductal carcinoma
1083	39	Caucasian	Moderate ductal hyperplasia
1084	71	Caucasian	Regressed Fibroadenoma
1085	34	Black	Breast Hamartoma
1086	50	Caucasian	Fibrocystic Disease - Multifocal sites of ductal hyperplasia
1087	62	Caucasian	Intraductal carcinoma
1088	51	Caucasian	Atypical Ductal Hyperplasia, Fibrocystic changes
1089	52	Caucasian	Stromal Fibrosis
1090	53	Caucasian	Invasive & In situ ductal carcinoma
1091	72	Caucasian	Nonproliferative fibrocystic changes
1092	62	Caucasian	Atypical ductal hyperplasia, intraductal papilloma

Table 3. Linear regression r^2 values with (significant p)

Data sets	Pathologic parameters	EXPANDED DATA Ultrasonic % Color Pixels		REDUCED DATA Ultrasonic % Color Pixels	
		Pre injection	Post injection	Pre injection	Post injection
All masses	Total MVA	0	0	0	0
$n_e = 50$	Total MVC	0	0	0	0
$n_r = 19$	MVA-5 ranges	0.15	0.25 (.02)	0.18	0.30 (.09)
	iMVD-5 ranges	0.09	0.14	0.03	0.32 (.06)
Benign	Total MVA	0	0	0	0
	Total MVC	0	0	0	0
$n_e = 30$	MVA-5 ranges	0.34 (.06)	0.49 (.003)	0.43	0.73 (.004)
$n_r = 10$	iMVD-5 ranges	0.13	0.19	0.05	0.36
Malignant	Total MVA	0	0	0	0
	Total MVC	0	0	0	0
$n_e = 20$	MVA-5 ranges	0.33	0.33	0.71 (.03)	0.59
$n_r = 9$	iMVD-5 ranges	0.62 (.009)	0.34	0.43	0.40

Table 4. T-statistic for significant variables: expanded data and % color pixels (p value)

Data set	Pathologic parameters	T – statistic value
All masses (post inj)	MVA by 30-39 μm Vessels	3.00 (.004)
Benign (post inj)	MVA by 30-39 μm Vessels	3.65 (.001)
	MVA by 40-49 μm Vessels	1.83 (.08)
Benign (pre inj)	MVA by 40-49 μm Vessels	2.18 (.04)
	MVA by 50μm & up Vessels	3.66 (.001)
Malignant (pre inj)	iMVD in 40-49 μm Range	3.95 (.001)

Table 5. T-statistic for significant variables: reduced data and % color pixels (p value)

Data Set	Pathologic Parameters	T – statistic Value
	MVA by 10-19 μm Vessels	1.90 (.07)
All masses (post inj)	MVA by 20-29 μm Vessels	2.05 (.05)
	MVA by 30-39 μm Vessels	2.00 (.06)
All masses (post inj)	iMVD in 30-39 μm Range	3.05 (.005)
	iMVD in 50 & up μm Range	2.60 (.02)
Benign (post inj)	MVA by 30-39 μm Vessels	3.72 (.003)
	MVA by 40-49 μm Vessels	1.76 (.01)
Malignant (pre inj)	MVA by 40-49 μm Vessels	4.01 (.003)

12. CAPTIONS

Figure 1. Color Doppler ultrasound of a benign breast mass (outlined in yellow) before (A) and after (B) contrast injection. Likewise for a malignant tumor pre (C) and post (D) contrast. Microscopic view (100x) of the benign (E) and malignant (F) mass showing microvessels stained with CD31 (arrows).

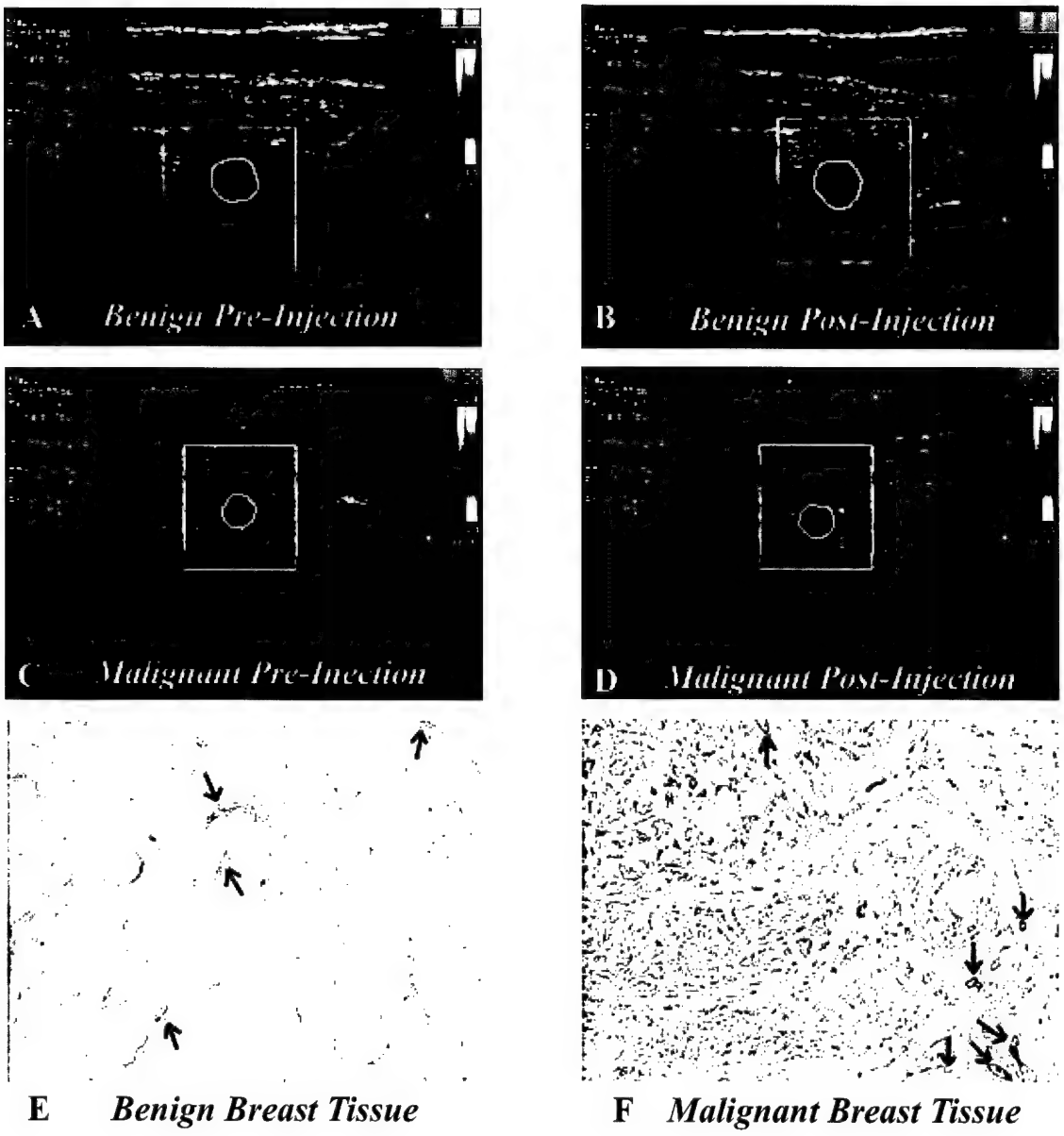

A Benign Pre-Injection
B Benign Post-Injection
C Malignant Pre-Inection
D Malignant Post-Injection
E Benign Breast Tissue
F Malignant Breast Tissue

Figure 1.

Appendix I

The Statement of Work from the original proposal:

Technical objectives 2 - 3
Task 1: Software development (months 1 - 6)

a. Develop image analysis software for the histomorphometry system to allow vessel distribution (i.e., histogram) and vessel density maps to be produced from 3D image data.

b. Develop 3D parameter extraction algorithms for the LIS 6000A system e.g., counting the number of interconnecting branches ("AV-shunts") and scoring the vessel tortuousity in collaboration with the consultant. Depending on the outcome of the statistical analysis, it is conceivable that new parameters will have to be extracted at a later date. Since the acquired 3D data volumes can be processed repeatedly this does not impact on the study design, it only demonstrates the flexibility of the data.

Technical objective 1
Task 2: Data collection (months 1 - 36)

a. recruit 50 patients per year. This is about half of the anticipated number of patients being enrolled in the NIH supported contrast study.

b. perform 3D CAI contrast studies as part of the already funded NIH project. This involves an extra injection of contrast (within the permitted total dose) and will add no more than 20 minutes to the total duration of the contrast study.

c. research coordinator to collect clinical information (pathology results, etc.).

Technical objectives 2 - 3
Task 3: Data analysis (months 6 - 36)

a. incorporate 3D imaging findings into the existing database (developed for the NIH supported contrast study).

b. quantitate 3D CAI results in collaboration with the consultant.

c. perform ROC analysis in collaboration with the statistician.

d. perform remaining statistical analysis in collaboration with the statistician.

Appendix II

Copy of article to appear in *Ultrasonics*, February 2000.

Session: Diagnostic Ultrasound Contrast Agents Version 1.1; June 24, 1999

Breast Tumor Vascularity Identified by Contrast Enhanced Ultrasound and Pathology: Initial Results

M.H. Chaudhari, F. Forsberg, A. Voodarla, F.N. Saikali, S. Goonewardene*, L. Needleman, W.T. Shi, G.C. Finkel*, B.B. Goldberg

Departments of Radiology and *Pathology, Thomas Jefferson University, Philadelphia, PA, USA.

Abstract: Quantifiable measures of vascularity obtained from contrast enhanced color flow images were correlated with pathologic vascularity measurements in ten female patients with a solid breast mass. Each patient received Levovist Injection® (Berlex Laboratories Inc., Montville, NJ). Color flow images pre and post contrast were obtained using an HDI 3000 unit (ATL, Bothell, WA) before removing the mass for pathologic vascularity assessments. Image processing techniques were used to obtain both the ultrasound and pathologic vascularity measurements. Multiple linear regression found significant correlations for ultrasonic vascularity measurements post contrast and pathology ($p = 0.02$ and 0.06). No correlations were found between pre contrast ultrasound and pathology. In conclusion, post contrast ultrasonic flow measures provide a noninvasive measure of breast tumor neovascularity. However, the patient population is small and until further patients are analyzed these conclusions are preliminary.

Keywords: Tumor neovascularity, image processing, color flow imaging, ultrasound contrast agents

Correspondence to: Flemming Forsberg, Ph.D.
Department of Radiology, Suite 763J, Main Building, Thomas Jefferson University
132 South 10th Street, Philadelphia, PA 19107, USA
Fax +1 - 215 - 955 8549; email: forsberg@esther.rad.tju.edu

1. Introduction

The goal of any breast imaging modality is to permit the early detection of tumors and to improve the differentiation between benign and malignant lesions, since this have been shown to significantly improve the chance of survival for patients with breast cancer [1]. Currently, the diagnosis of breast cancer is made with noninvasive techniques such as x-ray mammography followed by invasive techniques such as excisional or aspirational biopsies. However, in 33 % of patients, masses with no distinguishing features or with cystic/solid interiors cannot be diagnosed using mammography alone [2]. Moreover, the incidence of malignancy found by biopsy is very low ranging from 10 % to 35 % [2]. To improve the early characterization of breast massess, thereby reducing the number of benign breast tumors biopsied, and to treat breast tumors in the most effective manner the physiology of breast tumors must be understood.

Solid tumor growth and metastasis are dependent on angiogenesis, a process where blood vessels are sprouted from preexisting microvessels [3-4]. Tumor angiogenesis or neovascularity has been suggested to provide prognostic information on tumor malignancy [4]. It can be assessed invasively by the intratumoral MicroVessel Density (iMVD), which is an independent pathologic predictor of tumor angiogenesis and tumor state [4-7]. Ultrasound imaging is currently an auxiliary modality in breast imaging. Evaluation of the morphology of breast tumor vascularity with contrast enhanced Doppler ultrasound could provide a noninvasive diagnostic criterion similar to iMVD. To date, breast cancer diagnosis based on standard Doppler ultrasound flow detection has produced mixed results [8-10]. This may be due to the lack of sensitivity of Doppler techniques in detecting the small vessels and slow flow associated with tumor neovascularity. To improve the sensitivity and specificity of breast ultrasound intravenously administrated

microbubble based contrast agents, which produce up to 25 dB increase in blood flow signal intensities, have been used [11].

This study was conducted, to determine if quantifiable measures of lesion vascularity obtained from Doppler flow images pre and post contrast administration correlated with pathological assessments of vascularity; as a first step towards the non-invasive ultrasonic characterization of breast tumors.

2. Materials and Methods

Ten female patients with a solid breast mass diagnosed by x-ray mammography and who were scheduled for an excisional biopsy received Levovist Injection® (Berlex Laboratories Inc., Montville, NJ) intravenously at a dose volume of 10 ml, concentration of 300 mg/ml. Color flow images of the masses were obtained pre and post contrast using an HDI 3000 unit (ATL, Bothell, WA). Transaxial scans of the mass were performed at 5 levels, each encompassing 20 % of the cranio-caudal dimension. The color flow images at the point of maximal enhancement were recorded for each level and videotaped for further analysis. The Institutional Review Board of the university approved the study and all patients signed informed consent.

After removal of the mass, the specimens were sectioned in the same plane as the ultrasound images and stained with an endothelial cell marker, CD31, which targeted the microvessel walls staining them brown to dark brown in color. Finally, the sections were mounted onto 2"x 3" glass slides. The vascular morphology of the tissue, specifically, the number and area occupied by tumor vessels, were determined by a semi-automated histomorphometry system based on SMZ-10A microscope (Nikon, Melville, NJ) and ImagePro Plus software (Media Cybernetics, Silver Spring, MD). Using the software, microscope, and a Sony CCD camera, the entire

specimen area was captured and digitized under 100x magnification. For this study, 31 slides with a total of 5756 frames (dimensions: 640 pixels by 480 pixels or 1.27 mm^2) were examined. The number of color pixels on the color flow images relative to the pixel size of the mass can be used as a first order measure of mass vascularity [12]. Frozen ultrasound images were captured before and after contrast injection and digitized using the image analysis software. A total of 100 digitized scans were examined in this study.

Both the slide and video image analyses were patterned after chromaticity analysis methods used by Barbareschi et al. and Bell et al., respectively [13-14]. The aim of the slide image analysis was to develop a method in which only microvessels with lumen would be recognized by the software and, hence, counted and measured. Only vessels with lumen were chosen for three reasons: 1) the diameter of the vessels could be assessed directly, 2) other structures sporadically stained would not give false positives, and 3) a more repeatable and automated method could be performed. For each captured RGB color image of tumor area the slide image processing consisted of extracting vessel (saturation image) and tissue (blue image) enhanced images and performing mathematical morphometry to obtain an image on which automated count and measurements of microvessels was performed. For each slide the total microvessel area (MVA) and count (MVC) were determined and divided into five categories: vessel diameters between a) 10-19 μm, b) 20-29 μm, c) 30-39 μm, d) 40-49 μm, and e) 50 μm and above. The ultrasonic image processing consisted of extracting red, green, and blue images and performing mathematical morphometry to obtain an image only with color pixels. From the video analysis, the percent of color pixels within the mass was calculated for each level before and after contrast administration.

To determine if a linear relationship existed between pathologic and ultrasonic vascularity measurements, an equal number of data points must be assessed from both methods. Ten

ultrasonic data points per patient (5 pre and 5 post contrast; 50 in total) were consistently obtained because a hand held transducer was used and regardless of the size of the mass, scans at five levels of each mass could always be taken (albeit, sometimes with overlap between the levels). However, due to the size of some masses, five pathologic sections of a mass were sometimes not possible resulting in a total of 31 pathologic data points. Therefore, to be able to compare the data sets two solutions were developed, which ensured equal number of points in both data sets. One solution required ultrasonic data to be averaged and replaced until for each patient the number of scans equaled the number of pathology slides (i.e., data reduction). The other solution required pathologic data to be averaged and added to the number of slides for each patient until five data points were reached (i.e., data expansion). Statistical analysis was performed to determine if ultrasonic flow data (pre or post contrast) correlated with pathologic data in breast tumors. The linear relationship between ultrasonic and pathologic data was assessed using single and multiple variable linear regression techniques.

3. Results

Figure 1 shows sample digitized ultrasonic and pathologic images for both a benign and a malignant breast mass, while Figure 2 shows the corresponding vascularity maps depicting the number of vessels and a distribution of vessels by the five vessel ranges for both samples. There was a significant increase in the number of ultrasound color pixels pre to post contrast injection ($p < 0.003$). No statistical difference in ultrasonic color pixels was found between the benign and malignant masses. Table 1 gives the r^2 values for the linear regression for the reduced and expanded data sets. The multiple linear regression technique for the entire data set (i.e., benign and malignant lesions evaluated jointly) found significant correlations for ultrasonic color pixels

post injection with the percent of area in the five vessel ranges (expanded MVA; $p = 0.02$) and with the percent of vessels in the five vessel ranges (reduced iMVD; $p = 0.06$). No correlations were found between pre contrast ultrasonic color pixels and pathologic vascularity measurements using the entire data set.

To determine which variable in the multiple linear regression contributed the most, the T-statistic was evaluated for each variable. The significant T-values are listed for the reduced and expanded data sets in Tables 2 and 3, respectivley. For both the percent of area and percent of vessels in the five vessel ranges, the 30 to 39 μm vessel range contributed the most significantly to the linear relationship with the percent of color pixels post contrast injection, ($p = 0.004$ expanded and $p = 0.005$ reduced, respectively). Note, that the pre injection ultrasound vascularity measures from benign masses have the most significant contributions from larger vessels (≥ 40 μm) than the post injection ones (Table 2).

4. Conclusions

From the statistical analysis, it can be inferred that contrast enhanced color flow imaging provides some quantitative parameters, which correlate with direct pathologic vascularity assessments such as the iMVD. Specifically, the microvessel area and count for vessels 30 to 39 μm in diameter were most significant. This is in agreement with the qualitative observations of Burns et al. [15]. These results indicate that ultrasound imaging with contrast may produce a quantitative measure of the neovascularity within breast tumors; similar to results recently obtained with MRI [16]. However, the current patient population is very small and until further patients are analyzed, these conclusions are preliminary.

Acknowledgement

This work was supported in part by NIH-CA60854, DAMD17-97-1-7116, and Berlex Laboratories Inc., Montville, NJ, USA.

References

[1] Cancer Facts and Figures – 1997: Technical Report, Am. Cancer Soc. (1997).

[2] C.C. Kuni, Mammography in the 1990s: A plea for objective doctors and informed patients, Am. J. Prev. Med. 9 (1993) 185.

[3] J. Folkman, What is the evidence that tumors are angiogenesis dependent?, J. Natl. Cancer Inst. 82 (1990) 4.

[4] G. Gasparini, A. Harris, Clinical importance of the determination of tumor angiogenesis in breast carcinoma: much more than a new prognostic tool, J. Clin. Oncol. 12 (1995) 765.

[5] N. Weidner, J. Semple, W. Welch, Tumor angiogenesis and metastasis-correlation in invasive breast carcinoma, New Engl. J. Med. 324 (1991) 1.

[6] S. Bigler, M. Brawer, R. Deering, Neovascularization in carcinoma of the prostate: a quantitative morphometric study, Mod. Pathol. 5 (1992) 50.

[7] P. Macchiarini, G. Fontaini, et al., Relation of neovasculature to metastasis of non small-cell lung cancer, Lancet 340 (1992) 145.

[8] W-J. Lee, J-S Chu, et al., Breast cancer vascularity: color Doppler sonography and histopathology study, Breast Cancer Res. Treat. 37 (1996) 291.

[9] E. Sterns, S. SenGupta, F. Saunders, B. Zee, Vascularity demonstrated by Doppler ultrasound and immunohistochemistry in invasive ductal carcinoma of the breast, Breast Cancer Res. Treat., 40 (1996) 197.

[10] D. Cosgrove, J. Bamber, J. Davey, J. McKinna, H. Sinnett, Color Doppler signals from breast tumors, Radiology 176 (1990) 175.

[11] F. Forsberg, D.A. Merton, J.B. Liu, L. Needleman, B.B. Goldberg, Clinical applications of ultrasound contrast agents, Ultrasonics 36 (1998) 695.

[12] C.B. Meyerowitz, A.C. Fleischer, D.R. Pickens, G.B. Thurman, A.D. Borowsky, G. Thirsk, C.G. Hellerqvist, Quantification of tumor vascularity and flow with amplitude color Doppler sonography in an experimental model: preliminary results, J Ultrasound Med. 15 (1996) 827.

[13] M. Barbareschi, N. Weidner, et al., Microvessel density quantification in breast carcinomas, Appl. Immunohistochem. 3 (1995) 75.

[14] D. Bell, J. Bamber, R. Eckersley, Segmentation and analysis of color Doppler images of tumor vasculature, Ultrasound Med. Biol. 21 (1995) 635.

[15] P. Burns, J. Powers, D. Simpson, A. Brezina, A. Kolin, C. Chin, V. Uhlendorf, T. Fritzch, Harmonic power mode Doppler using microbubble contrast agents: an improved method for small vessel flow imaging, IEEE Ultrason. Symp. (1994) 1547.

[16] L. Esserman, N. Hylton, T. George, N. Weidner, Contrast-enhanced magnetic resonance imaging to assess tumor histopathology and angiogenesis in breast carcinoma, The Breast J. 5 (1999) 13.

Table 1. Linear regression r^2 values with (significant p)

Data sets	Pathologic parameters	EXPANDED DATA Ultrasonic % Color Pixels		REDUCED DATA Ultrasonic % Color Pixels	
		Pre injection	Post injection	Pre injection	Post injection
All masses	Total MVA	0	0	0	0
$n_e = 50$	Total MVC	0	0	0	0
$n_r = 19$	MVA-5 ranges	0.15	0.25 (.02)	0.18	0.30 (.09)
	iMVD-5 ranges	0.09	0.14	0.03	0.32 (.06)
Benign	Total MVA	0	0	0	0
	Total MVC	0	0	0	0
$n_e = 30$	MVA-5 ranges	0.34 (.06)	0.49 (.003)	0.43	0.73 (.004)
$n_r = 10$	iMVD-5 ranges	0.13	0.19	0.05	0.36
Malignant	Total MVA	0	0	0	0
	Total MVC	0	0	0	0
$n_e = 20$	MVA-5 ranges	0.33	0.33	0.71 (.03)	0.59
$n_r = 9$	iMVD-5 ranges	0.62 (.009)	0.34	0.43	0.40

Table 2. T-statistic for significant variables: expanded data and % color pixels (p value)

Data set	Pathologic parameters	T – statistic value
All masses (post inj)	MVA by 30-39 μm Vessels	3.00 (.004)
Benign (post inj)	MVA by 30-39 μm Vessels	3.65 (.001)
	MVA by 40-49 μm Vessels	1.83 (.08)
Benign (pre inj)	MVA by 40-49 μm Vessels	2.18 (.04)
	MVA by 50μm & up Vessels	3.66 (.001)
Malignant (pre inj)	iMVD in 40-49 μm Range	3.95 (.001)

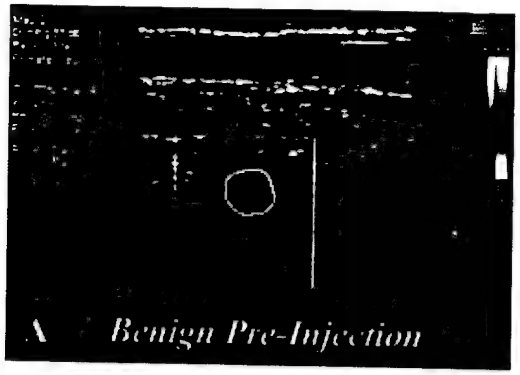
Table 3. T-statistic for significant variables: reduced data and % color pixels (p value)

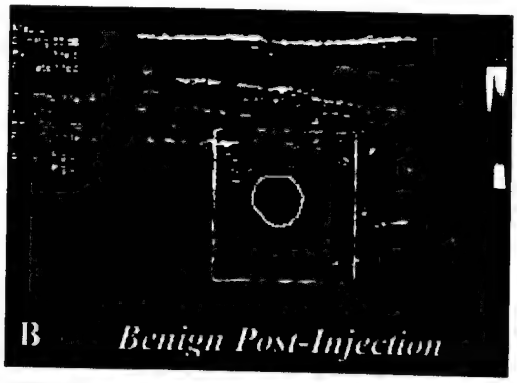
Data Set	Pathologic Parameters	T – statistic Value
	MVA by 10-19 μm Vessels	1.90 (.07)
All masses (post inj)	MVA by 20-29 μm Vessels	2.05 (.05)
	MVA by 30-39 μm Vessels	2.00 (.06)
All masses (post inj)	iMVD in 30-39 μm Range	3.05 (.005)
	iMVD in 50 & up μm Range	2.60 (.02)
Benign (post inj)	MVA by 30-39 μm Vessels	3.72 (.003)
	MVA by 40-49 μm Vessels	1.76 (.01)
Malignant (pre inj)	MVA by 40-49 μm Vessels	4.01 (.003)

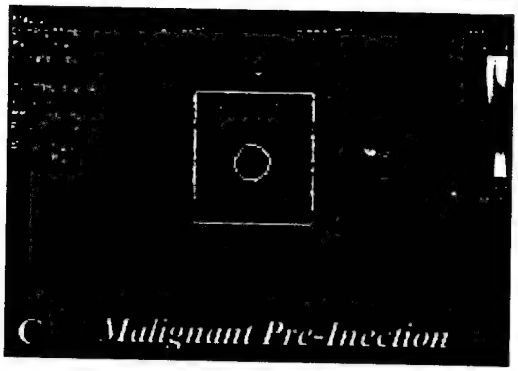
Captions

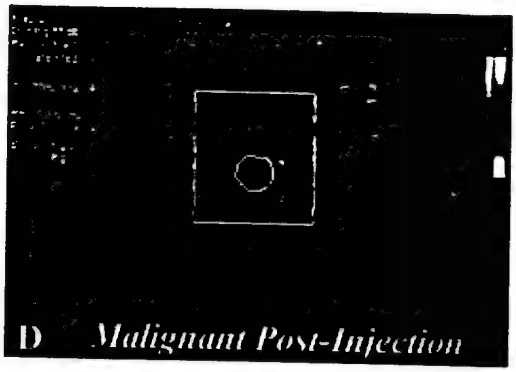
Figure 1. Color Doppler ultrasound of a benign breast mass (outlined in yellow) before (A) and after (B) contrast injection. Likewise for a malignant tumor pre (C) and post (D) contrast. Microscopic view (100x) of the benign (E) and malignant (F) mass showing microvessels stained with CD31 (arrows).

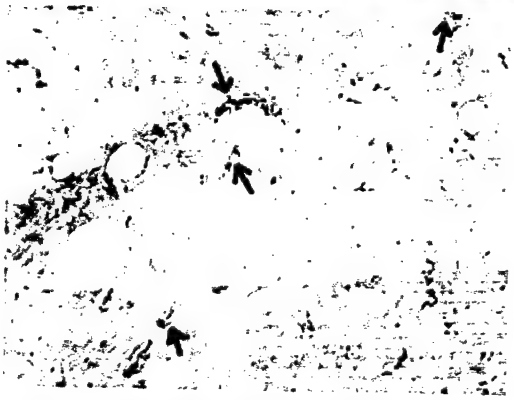
Figure 2. A vascularity map with the number of vessels per image and their distribution within the five ranges for the benign (A & B) and malignant (C & D) mass, respectively.











E ***Benign Breast Tissue***

F ***Malignant Breast Tissue***

Chaudhari et al. Fig. 1

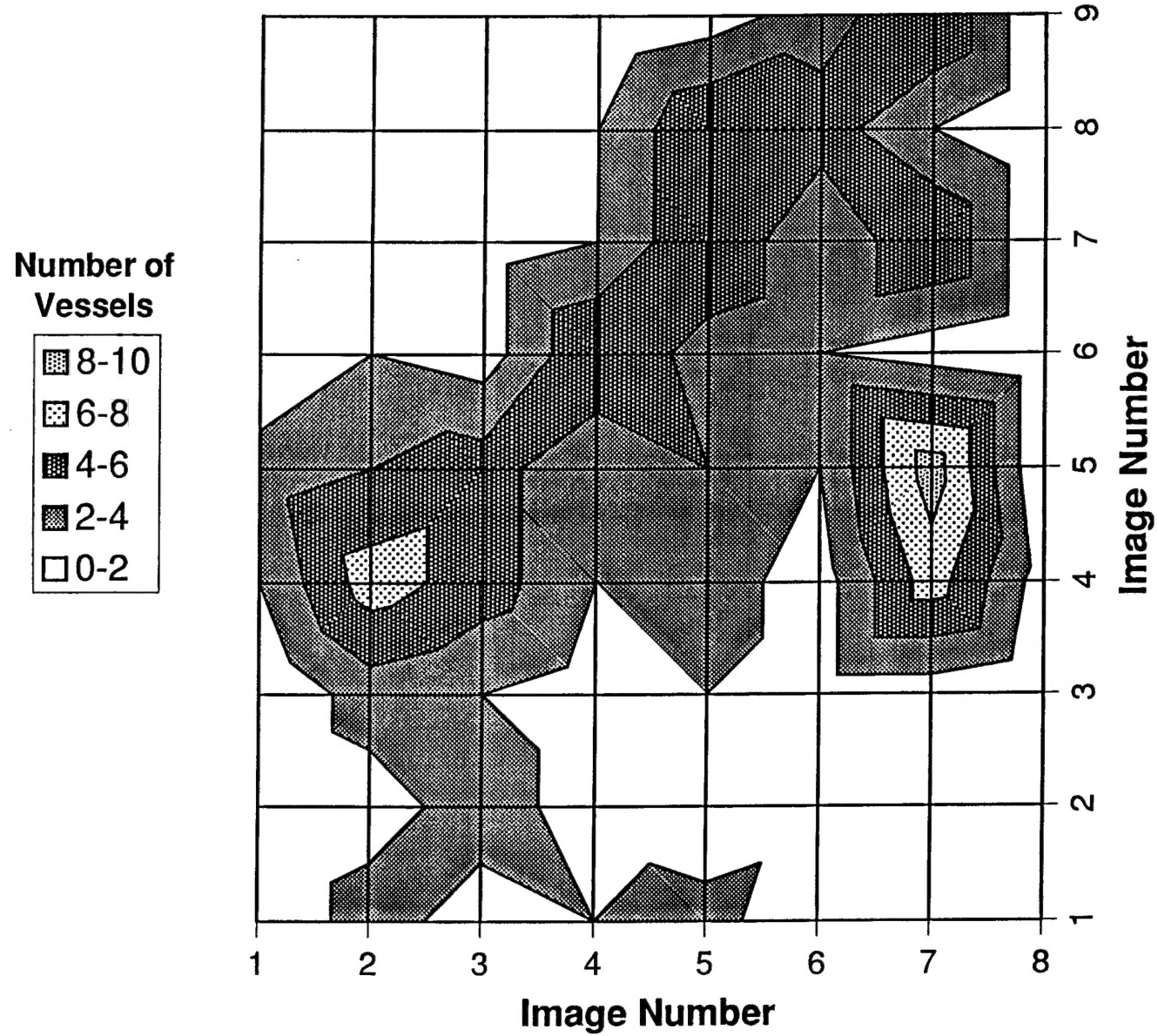

Number of Vessels
8-10
6-8
4-6
2-4
0-2
1
2
3
4
5
6
7
8
Image Number
9
8
7
6
5
4
3
2
1
Image Number

Figure 2A

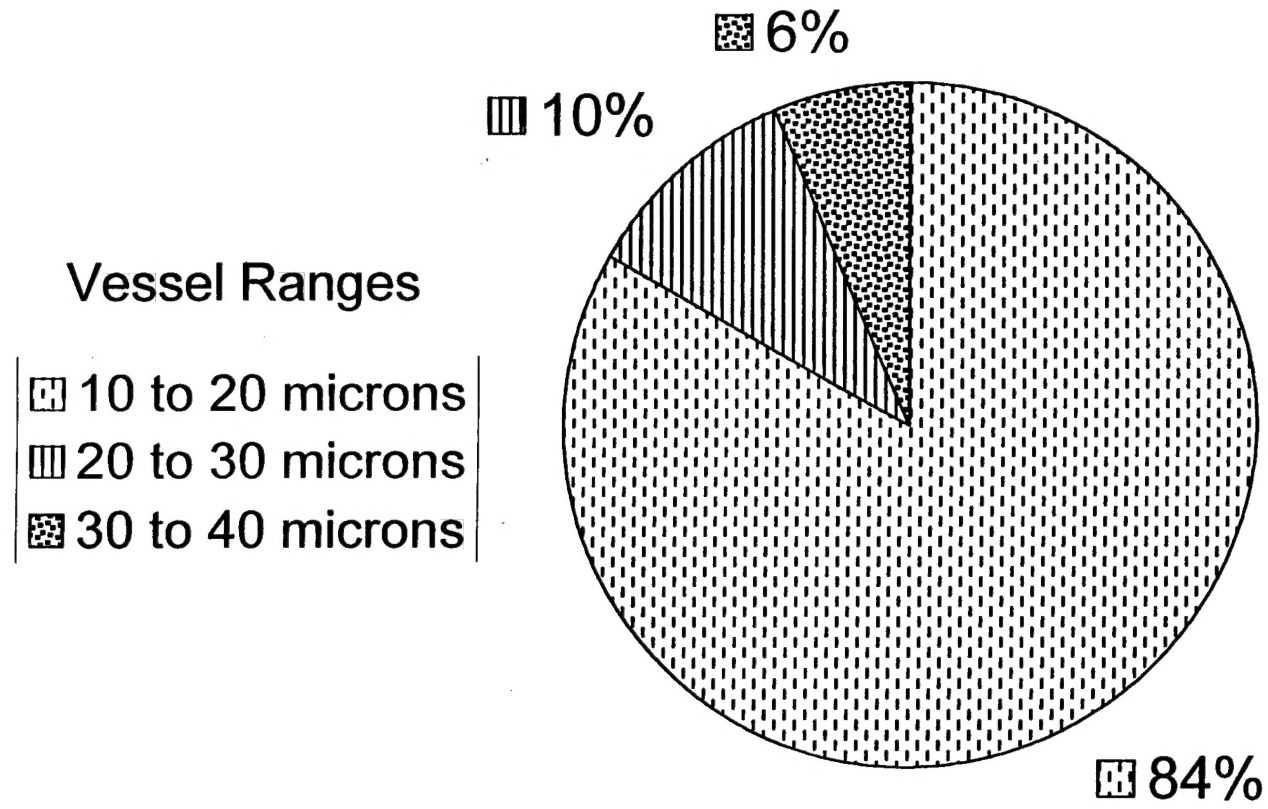

6%
10%
Vessel Ranges
10 to 20 microns
20 to 30 microns
30 to 40 microns
84%

Figure 2B

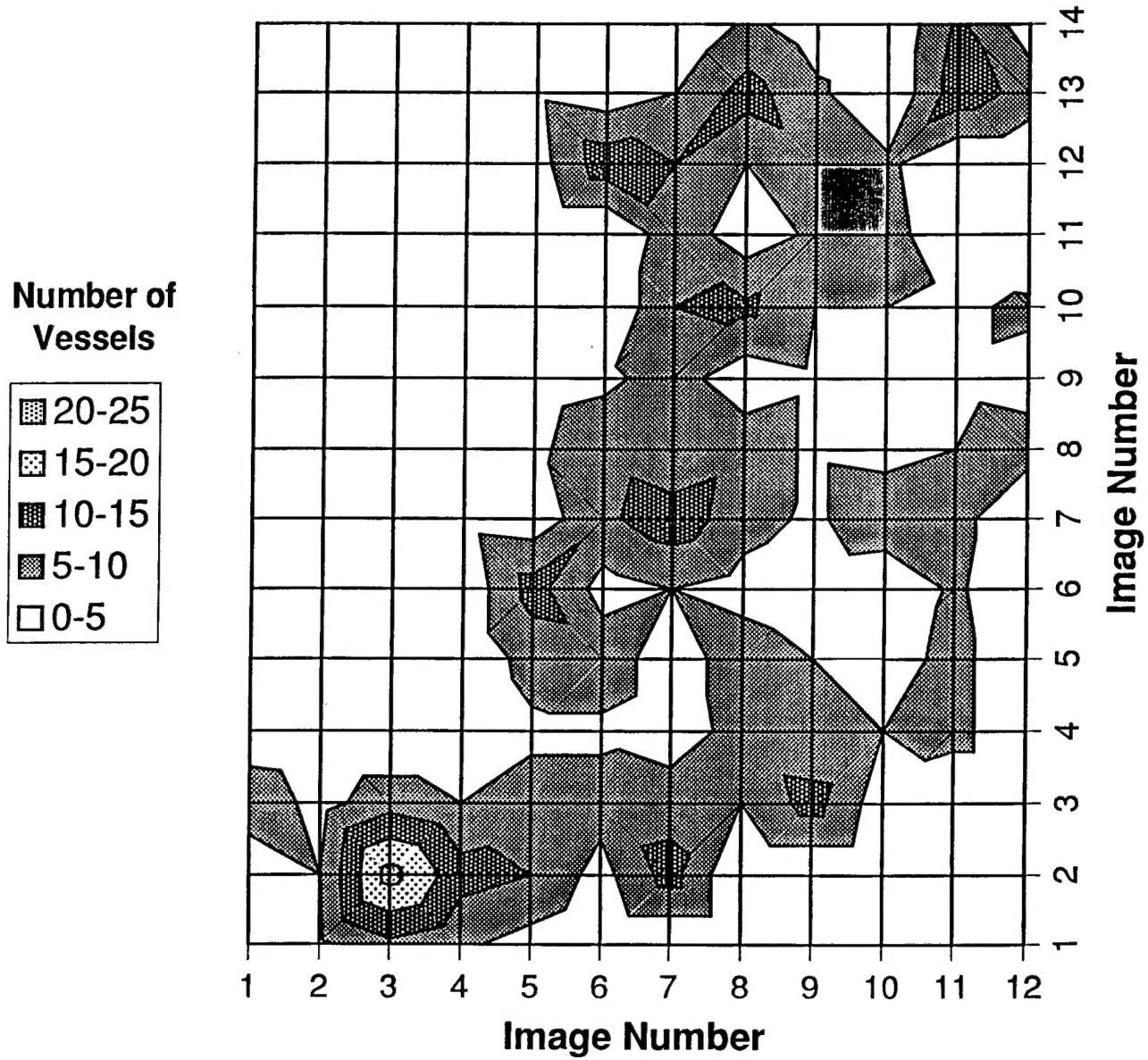

Number of Vessels
20-25
15-20
10-15
5-10
0-5
1 2 3 4 5 6 7 8 9 10 11 12
Image Number
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Image Number

Figure 2C

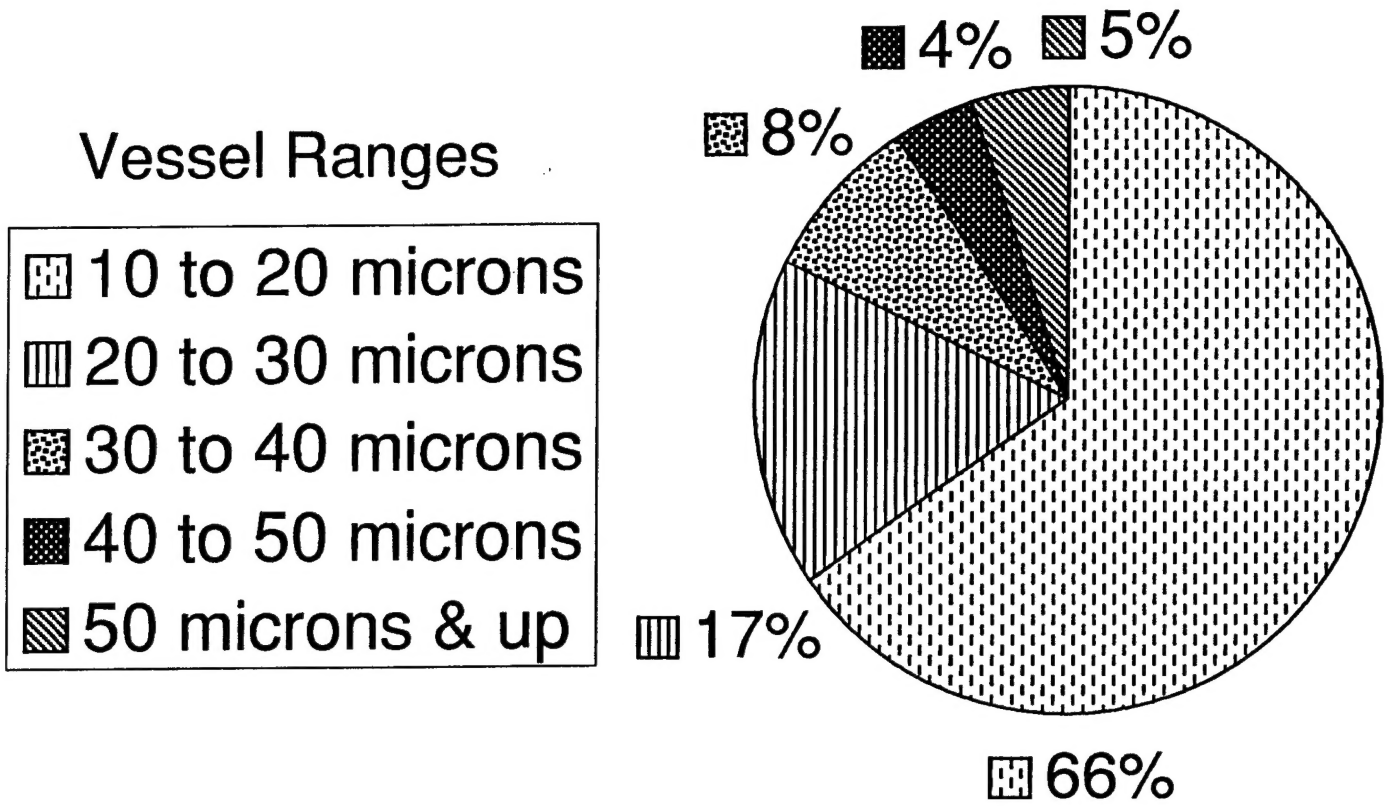

Vessel Ranges
10 to 20 microns
20 to 30 microns
30 to 40 microns
40 to 50 microns
50 microns & up
4%
5%
8%
17%
66%

Figure 2D